Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Identification and virulence of *Chryseobacterium indologenes* isolated from diseased yellow perch (*Perca flavescens*)

J.W. Pridgeon, P.H. Klesius and J.C. Garcia

Aquatic Animal Health Research Unit, USDA-ARS, Auburn, AL, USA

Keywords
Chryseobacterium indologenes, pathogen, virulence, yellow perch.

Correspondence
Julia W. Pridgeon, Aquatic Animal Health Research Unit, USDA-ARS, 990 Wire Road, Auburn, AL 36832, USA. E-mail: julia.pridgeon@ars.usda.gov

2013/1868: received 17 October 2012, revised 2 November 2012 and accepted 5 November 2012

doi:10.1111/jam.12070

Abstract

Aim: To identify pathogen of diseased yellow perch and determine their virulence.

Methods and Results: Fifteen Gram-negative bacterial isolates were recovered from the skin lesions of diseased yellow perch (*Perca flavescens*). Based on API 20NE test, ten isolates were found to share 67·2–99·9% homologies with *Chryseobactertium indologenes*. Based on fatty acid methyl ester analysis, 13 isolates were found to share similarities with *C. indologenes* and other species of *Chryseobacterium*. Based on sequencing results of partial 16S rRNA gene, 13 isolates shared 99% identities (e value = 2e−50) with the 16S rRNA sequence of *C. indologenes* (GenBank HQ259684). Based on the 16S–23S rRNA intergenic spacer region (ISR) sequence, the 13 isolates shared 88% identity (e value = 1e−165) with the 16S–23S ISR sequence of *C. indologenes* (GenBank EU014570). T-coffee multiple sequence alignment revealed that the partial 16S rRNA or the 16S–23S ISR sequence of the 13 isolates shared 100% identity with each other. When healthy yellow perch were exposed to the 15 isolates by bath immersion (*c.* 6×10^7 CFU ml^{-1} for 1 h), only *C. indologenes* isolates killed 10–20% of fish, whereas other isolates were avirulent. When yellow perch were exposed to *C. indologenes* by intraperitoneal injection, mortality was dose dependent, with LD_{50} and LD_{95} values of $1{\cdot}5 \times 10^8$ and $3{\cdot}2 \times 10^8$ CFU per fish, respectively.

Conclusions: *Chryseobactertium indologenes* could be pathogenic to yellow perch.

Significance and Impact of the Study: This is the first report on the isolation of *C. indologenes* from diseased yellow perch. Virulence studies suggested that *C. indologenes* could become pathogenic to yellow perch.

Introduction

The genus *Chryseobacterium* belongs to the family of *Flavobacteriaceae*. Several bacteria formally classified in the genus *Flavobacterium* were reclassified within the genus *Chryseobacterium* according to Vandamme *et al.* (1994), including *Flavobacterium balustinum*, *Flavobacterium gleum*, *Flavobacterium indologenes*, *Flavobacterium indoltheticum*, *Flavobacterium meningosepticum* and *Flavobacterium scophthalmum*. Members of the genus *Chryseobacterium* are widely distributed in various environments, including soil (Benmalek *et al.* 2010), plants (Cho *et al.* 2010), fresh water (Kim *et al.* 2008; Park *et al.* 2008), marine environment (de Beer *et al.* 2006), wastewater (Yoon *et al.* 2007) and food sources, such as milk (Hantsis-Zacharov *et al.* 2008) and chicken (de Beer *et al.* 2005). Several species of *Chryseobacterium* have been isolated from diseased fish. For example, *Chryseobacterium scophthalmum* was isolated from diseased turbot (*Scophthalmus maximus*) in Scotland (Mudarris *et al.* 1994); *Chryseobacterium shigense* was isolated from rainbow trout (*Oncorhynchus mykiss*) during disease

outbreaks in Spain (Zamora *et al.* 2012); *Chryseobacterium piscicola* was reported to be responsible for mortality in farmed Atlantic salmon (*Salmo salar*) in Chile (Ilardi and Avendaño-Herrera 2008; Ilardi *et al.* 2009) and Finland (Ilardi *et al.* 2010). In addition, increasing incidents of *Chryseobacterium* infections have been reported in hospital environments (Hsueh *et al.* 1997; Bhuyar *et al.* 2012).

In January of 2012, mortality among yellow perch was observed in a US fish farm, with symptoms of gross skin lesions below the dorsal fin as well as the caudal peduncle. Diseased fish were sent to Aquatic Animal Health Research Unit, USDA-ARS for pathogen identification. After culturing the skin lesions on Shieh agar plates supplemented with tobramycin (Decostere *et al.* 1997), majority of the colonies showed yellow pigments resembling the appearance of *Flavobacterium*, whereas a few of them had no pigment (white colonies). A total of 15 colonies (yellow and white) were then randomly picked from the plates and subjected to bacterial identification. However, at that stage, it was unknown whether the bacteria were pathogenic to yellow perch. Therefore, the objectives of this study were (i) to conduct biochemical identifications of the 15 isolates cultured from the skin lesions of the diseased yellow perch; (ii) to determine whether any of these colonies could cause mortality to yellow perch by bath immersion; (iii) to perform molecular identifications for the isolates that were able to cause mortality among yellow perch by bath immersion; and (iv) to determine the virulence (LD_{50} value) of the isolate to yellow perch by intraperitoneal injection.

Materials and methods

Bacterial isolates and culture conditions

After mortality was observed in a US yellow perch farm in January 2012, four diseased moribund yellow perch (*Perca flavescens*) were collected, freshly frozen and sent to the Aquatic Animal Health Research Laboratory, USDA, ARS within 24 h on ice. Fifteen bacterial isolates were recovered from skin lesions of the four diseased fish. Skin lesions on diseased fish were photographed. Based on the symptoms of gross skin lesions below the dorsal fin as well as the caudal peduncle, columnaris disease was suspected. Skin lesion samples were washed in sterile phosphate-buffered saline three times to minimize contamination. A sterile loop was then used to inoculate the washed skin lesion samples onto Shieh agar plates containing 1 μg ml^{-1} of tobramycin according to Decostere *et al.* (1997). After overnight growth at 19 $\pm$ 1°C, majority of the colonies on the culture plates appeared yellowish, resembling *Flavobacterium columnare*, whereas a few colonies grew on the plates appeared white. A total of 15 colonies (13 yellow and 2 white) were then randomly picked and restreaked onto Shieh agar plates containing 1 μg ml^{-1} of tobramycin to obtain single colonies. Single-colony bacterial cultures grown in modified Shieh medium were then subjected to biochemical and molecular identification. Glycerol stock (10% glycerol) of each isolate was prepared in modified Shieh medium and stored at −80 C.

Gram staining, oxidase test, catalase test and API 20 NE test

Gram staining was performed with Gram staining kit and reagents followed the instruction of the manufacturer (Becton Dickinson, Franklin Lakes, NJ, USA). Oxidase test was performed by adding bacterial smear to filter paper containing BactiDrop oxidase reagent (Remel, Lenexa, KS, USA). Colour development was observed within 1 min. Catalase test was performed by adding a drop of hydrogen peroxide (Fisher Scientific, Pittsburgh, PA, USA) to a slide that contained live bacterial smear. API 20 NE bacterial identification was performed according to manufacturer's instruction (bioMérieux, Durham, NC, USA).

Microbial identification using fatty acid methyl ester profiling

Fatty acid methyl ester (FAME) profiling was performed according to manufacturer's instruction (MIDI Labs, Newark, DE, USA). Briefly, overnight bacterial cultures (25–30 mg) were transferred to 13 × 100 mm glass tubes. Bacterial cells were saponified in 3·75 N NaOH in 50% methanol for 30 min, followed by methylation with 3·25 N HCl in methanol for 10 min. FAMEs were then mixed with hexane and methyl tert-butyl ether (1 : 1) for 10 min. After brief centrifugation, the top phase was collected and mixed with 0·3 N NaOH to remove any free fatty acids and residual extraction solvent. After brief centrifugation, the top phase was removed and subjected to gas chromatography using Agilent 6850 GC system (Agilent Technology, Santa Clara, CA, USA). FAME profiles were then compared to FAME profiles deposited in the standard RCLN50 library, and the similarity indices were then calculated by Sherlock Library Search (MIDI, Newark, DE, USA). An exact match of the fatty acid make-up of an unknown sample with that of a known library entry would result in a similarity index of 1·000. Samples with a similarity of 0·500 or higher with a separation of 0·100 between the first and second choice are considered good library comparison.

Virulence of the 15 isolates to yellow perch by bath immersion

To study the virulence of the 15 bacterial isolates to yellow perch, isolates were cultured overnight in Shieh medium supplemented with 1 μg ml^{-1} of tobramycin at 19 ± 1°C. The concentration (CFU ml^{-1}) of each isolate was determined through serial dilutions. Briefly, optical density (OD) of the bacterial cultures was measured at 540 nm using a Thermo Spectronic spectrophotometer (Fisher Scientific, Pittsburgh, PA, USA) and adjusted to OD = 1. Serial dilutions were prepared in Shieh medium, and 100 μl of diluted bacterial culture was plated onto Shieh agar plates. After 24-h incubation at 19 ± 1°C, the average number of CFU ml^{-1} was calculated for each isolate. Healthy yellow perch (2 ± 0·5 g) were obtained from a US yellow perch supplier and acclimated for 30 days prior to challenge. The health status (culture negative) of the yellow perch prior to challenge was evaluated by randomly sampling of selected fish and culturing skin and kidney tissue on Shieh agar plates. Acclimated fish were maintained in 57 l glass aquaria (10 fish per tank) with flow-through dechlorinated tap water and constant aeration with water temperature at 19 ± 1°C. The flow-through water was at a rate of 0·5 l min^{-1}. Dissolved oxygen range was 6 to 8 mg l^{-1}. A 12 : 12 h light/dark schedule was maintained. Fish were fed daily with commercial Aquamax Grower (Brentwood, MO, USA) at 4% of their body weight. For bath immersion, bacteria at beginning concentration of approximately 6 × 10^7 CFU ml^{-1} (100 ml of $OD_{540\ nm}$ = 1·0 culture was added to 900 ml of water) was used for duration of 1 h in 2 l beakers. After the 1-h immersion, an aliquot of water sample was used to check the viability and the concentration of the bacteria. After the 1-h immersion, fish were returned to 57 l glass aquaria (10 fish per tank, 3 tanks per isolate). Mortalities were recorded daily for 21 days postexposure. For 13 *Chryseobacterium indologenes* isolates identified by FAME and sequencing, bath immersion was not repeated because these isolates shared 100% identity with each other at the 16S-23S rRNA intergenic spacer region (ISR) region. For non-*Chryseobacterium* isolates, bath immersions were repeated three times. The anterior kidney of dead fish was cultured on Shieh agar plates for bacterial isolation followed by API 20 NE test. Mean cumulative mortality data were analysed by analysis of variance (ANOVA) using SIGMASTAT statistical analysis software (Systat Software, San Jose, CA, USA).

Primer design, polymerase chain reaction and sequencing of 16S-23S rRNA ISR

Based on mortality results, only yellow-pigmented bacterial isolates identified as *Chryseobacterium* spp. showed virulence to yellow perch, whereas other white-pigmented isolates were avirulent to yellow perch. To determine whether these *Chryseobacterium* isolates were identical, a forward primer Chry-16S-F (5′-GCATCAGCCATGGCGCGGTG-3′) located at 1327 to 1347 bp of the complete 16S rRNA sequence of *Chryseobacterium* sp. (GenBank accession no. JQ740255) was designed based on the fact that it was conserved among different species of *Chryseobacterium*. Similarly, a reverse primer Chry-23S-R (5′-CCTTAACGATTTCTTTCCTA-3′) located at 807–787 bp of the 23S sequence of *C. piscicola* (GenBank accession no. HM131451) was also designed. Polymerase chain reaction (PCR) was performed in a 25 μl mixture consisting of 12·5 μl of 2 × Taq PCR Master Mix (Qiagen, Valencia, CA, USA), 9·5 μl of nuclease-free H_2O, 1 μl of genomic DNA (10 ng ul^{-1}), 1 μl of forward primer (5 umol l^{-1}) and 1 μl of reverse primer (5 umol l^{-1}). PCR was carried out in a Biometra T Gradient thermocycler (Biometra, Goettingen, Germany). PCR conditions consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of 45 s at 94°C, 45 s at 53°C and 2 min at 72°C, followed by a final extension of 10 min at 72°C. PCR products were analysed by 1% agarose gel by electrophoresis. Individual single bands were excised and purified using gel purification kit according to the manufacturer's protocol (Qiagen). Purified PCR products were sequenced using Chry-16S-F primer at the USDA-ARS Mid South Genomic Laboratory (Stoneville, MS, USA) on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequence analysis

Sequences were analysed using the National Center for Biotechnology Information (NCBI) BLAST program to search for sequence identities. Multiple sequence alignment for partial 16S rRNA and 16S-23S ISR sequences was performed using T-coffee method (http://tcoffee.crg.cat/apps/tcoffee/do:regular) (Notredame *et al.* 2000; Di Tommaso *et al.* 2011).

Determination of LD_{50} of *Chryseobactertium indologenes* to yellow perch by intraperitoneal injection

BLASTN analysis revealed that all *Chryseobacterium* isolates shared 99% identities with the 16S rRNA sequence of *C. indologenes* strain B7 (GenBank HQ259684). T-coffee multiple sequence alignment revealed that the partial 16S rRNA or 16S-23S ISR sequence of the 13 isolates shared 100% identities with each other. T-coffee multiple sequence alignment analysis revealed that all isolates of *Chryseobacterium* shared 100% homology with each other. Therefore, only a single isolate (isolate 4) was

chosen to determine the LD_{50} value of *C. indologenes* to healthy yellow perch by intraperitoneal injection. After overnight incubation of isolate 4 at 19 ± 1°C, the average number (CFU ml^{-1}) was calculated. Five different doses (ranging from 6×10^7 to 3×10^8 CFU per fish) that killed 0–100% fish were administered to anaesthetized (10 mg l^{-1} of tricaine methanesulphonate) yellow perch (2 ± 0·5 g) through intraperitoneal injection. The experiments were repeated three times. After exposing yellow perch to isolate 4, mortalities were recorded daily for 21 days postexposure. The presence of *C. indologenes* in dead fish was determined by culturing anterior kidney samples on Shieh agar plates followed by API 20 NE biochemical test. LD_{50} values were calculated using POLOPLUS probit and logit analysis software (LeOra Software, Petaluma, CA, USA).

Results

Collection of bacterial isolates from diseased yellow perch

The lesions of the diseased yellow perch appeared yellowish, with very strong smell. Diseased yellow perch had symptoms of gross skin lesions below the dorsal fin as well as the caudal peduncle (Fig. 1). Majority (13/15) of the colonies appeared yellowish, whereas two colonies (isolate 8 and 9) were white.

Gram staining, oxidase test, catalase test and API 20 NE test

All isolates comprised Gram-negative catalase and oxidase-positive rods/cells that produced β-glucosidase hydrolysis (esculin ferric citrate/ESC as substrate) and protease hydrolysis (gelatin/GEL as substrate). Based on API 20NE test results, ten isolates were found to share 67·2% to 99·9% homologies with *C. indologenes*. Of the 15 isolates, ten were found to share identities with *C. indologenes*. Three were found to share similarities with *Empedobacter brevis*.

FAME analysis of the bacterial isolates

FAME analysis revealed that 13 of the 15 isolates shared similar profiles with that of *Chryseobacterium* species deposited at the RCLN50 database, with first choice similarity indices ranging from 0·246 to 0·395. The 13 isolates had four major fatty acid peaks (Fig. 2a). The biggest peak was 15:0 iso fatty acid at retention time of 2·325 min, which accounted for 40% of the total FAME response. The second biggest peak was summed feature 4 (10 methyl 16:0 and 17:1 iso w9c) at retention time of 2·892 min, which was 28% of the total response. The other two peaks that shared similar percentage (12%) of total response were summed feature 3 (16:1 w7c and 16:1 w6c at retention time of 2·713 min) and 17:0 iso 3OH

Figure 1 Skin lesions of diseased yellow perch, *Perca flavescens*. (a) Whole body picture; (b) skin lesions under the dorsal fin; (c) skin lesions at the caudal peduncle area.

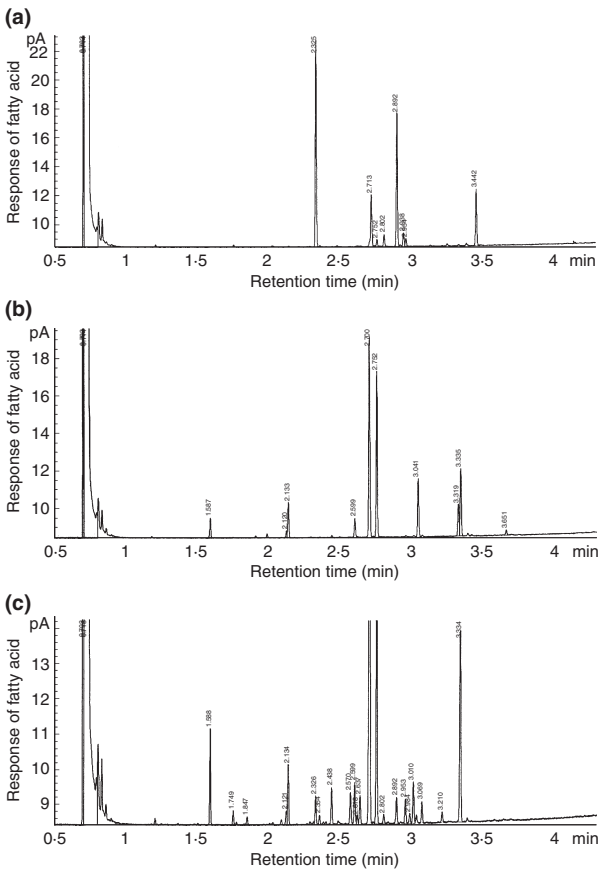

Figure 2 Fatty acid methyl ester (FAME) profiles of bacterial isolates cultured from skin lesions of diseased yellow perch. (a) Representative FAME profile of the 13 isolates identified as *Chryseobacterium* species; (b) FAME profile of isolate 8; (c) FAME profile of isolate 9.

(at retention time of 3·442 min) (Fig. 2a). Fatty acid profile of isolate 8 had eight major peaks (Fig. 2b), whereas that of isolate 9 had 14 major peaks (Fig. 2c). Both isolate 8 and isolate 9 shared some similarities with *Aeromons veronii*, with similarity indices of 0·285 and 0·474, respectively.

Virulence of the bacterial isolates to yellow perch by bath immersion

Virulence of the 15 bacterial isolates to yellow perch by bath immersion is summarized in Table 1. At the end of the 1-h immersion, water samples were culture positive as input bacteria (*Chryseobacterium* or *Aeromonas*) and the concentrations were slightly smaller (4×10^7 CFU ml^{-1}) than the input concentration (6×10^7 CFU ml^{-1}). The isolates that killed 10% or 20% yellow perch were *Chryseobacterium*. Dead fish were all culture positive on Shieh agar plates. Colonies collected from the posterior kidney of freshly dead yellow perch after bath immersion with isolate 4 were identified as *C. indologenes* by FAME analysis, with similar index of 0·701. Neither isolate 8 nor isolate 9 was found to be virulent to yellow perch by bath immersion (Table 1).

Table 1 Virulence of the 15 isolates to yellow perch by bath immersion

Isolate no.	Immersion concentration (CFU ml^{-1})	Mortality (%)
Medium control	0	0
1	$6{\cdot}7 \times 10^7$	10
2	$7{\cdot}7 \times 10^7$	10
3	$7{\cdot}4 \times 10^7$	10
4	$3{\cdot}4 \times 10^7$	20
5	$4{\cdot}0 \times 10^7$	0
6	$3{\cdot}7 \times 10^7$	10
7	$4{\cdot}7 \times 10^7$	0
8	$6{\cdot}9 \times 10^7$	0
9	$8{\cdot}6 \times 10^7$	0
10	$7{\cdot}2 \times 10^7$	0
11	$5{\cdot}4 \times 10^7$	10
12	$9{\cdot}2 \times 10^7$	0
13	$9{\cdot}4 \times 10^7$	0
14	$8{\cdot}6 \times 10^7$	0
15	$9{\cdot}2 \times 10^7$	0

PCR and sequencing results

Using Chry-16S-F and Chry-23S-R primers and gDNA of the 15 isolates as templates, a single sharp band of *c.* 800 –1000 bp (Fig. 3) showed up in all 13 isolates identified as *Chryseobacterium* species by FAME. Based on sequencing results of partial 16S rRNA gene, 13 isolates shared 99% identities (e value = 2e−50) with the region from 1352 to 1469 bp of the 16S rRNA sequence of *C. indologenes* strain B7 (GenBank HQ259684), with only one bp deletion at the 1364 bp of the sequence of HQ259684 (Fig. 4). However, the 13 isolates shared 100% identities with each other (Fig. 4). Based on 16S-23S rRNA ISR sequence, the 13 isolates shared 88% identities (430/489, e value = 1e−165) with the 16S-23S ISR sequence of *C. indologenes* strain ATCC 29897 (GenBank EU014570). T-coffee multiple sequence alignment revealed that the 16S-23S ISR sequences of the 13 isolates shared 100% identities with each other. The sequence of 860 bp of the partial 16S and ISR of these *C. indologenes* was deposited at GenBank with accession no. JX649950.

LD_{50} of *Chryseobactertium indologenes* to yellow perch by intraperitoneal injection

Based on sequencing results and T-coffee multiple sequence alignment, the 13 isolates shared 100%

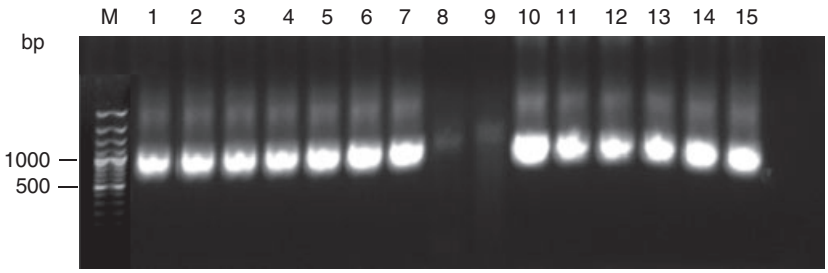

Figure 3 Electrophoresis of polymerase chain reaction (PCR) products on 1% agarose gel. M: 100 bp DNA marker. Lane 1–15 were PCR products using genomic DNA from isolate 1–15 as template, respectively.

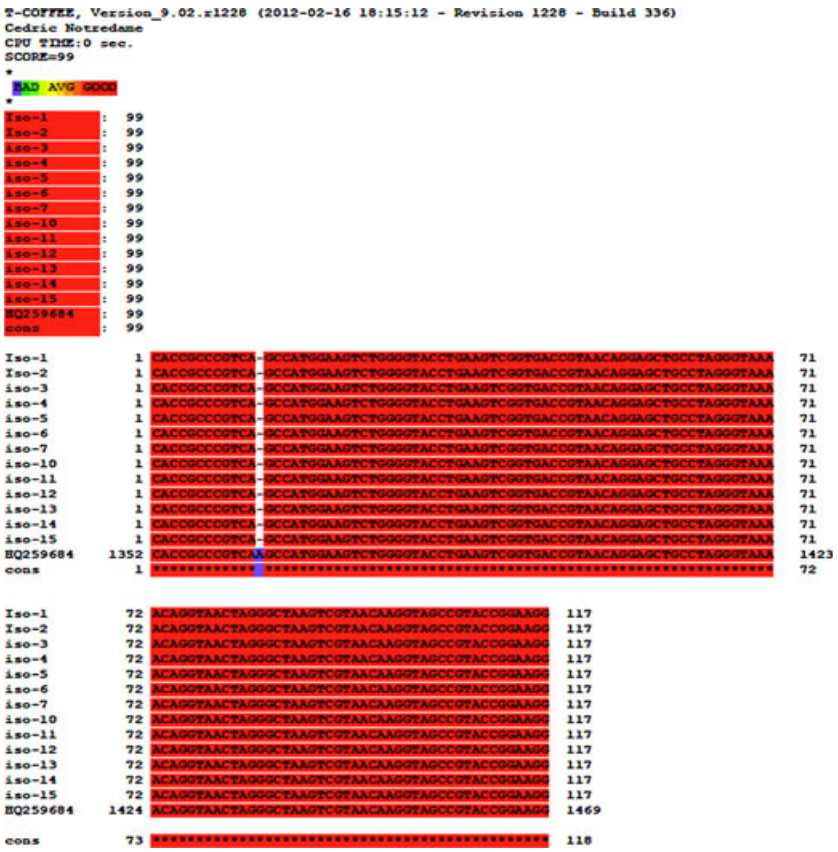

Figure 4 T-coffee multiple sequence alignment of the partial 16S RNA of the 13 *Chryseobacterium* isolates with *Chryseobactertium indologenes* strain B7 (HQ259684).

identities with each other at the partial 16S rRNA region or the 16S-23S ISR region of *C. indologenes*, suggesting that all 13 isolates represented essentially the same species. Therefore, only one isolate (isolate 4) was chosen for the injection study. The LD_{50} value of isolate 4 to yellow perch ($2 \pm 0{\cdot}5$ g) was $1{\cdot}5 \times 10^8$ CFU per fish, with 95% confidence intervals ranging from $1{\cdot}2 \times 10^8$ to $1{\cdot}8 \times 10^8$ CFU per fish. The LD_{95} value of isolate 4 to yellow perch ($2 \pm 0{\cdot}5$ g) was $3{\cdot}2 \times 10^8$ CFU per fish, with 95% confidence intervals ranging from $2{\cdot}5 \times 10^8$ to $5{\cdot}1 \times 10^8$ CFU per fish. After yellow perch were exposed to *C. indologenes* by intraperitoneal injections, the fish showed a loss of appetite and balance. Another obvious sign presented in diseased fish injected with *C. indologenes* was a swelling above the anal fin, which was also observed in diseased fish collected from the farm (Fig. 1).

Discussion

Fifteen Gram-negative bacteria isolates were cultured from the skin lesions of yellow perch collected from a disease outbreak in January 2012. Based on API 20 NE bacterial identification test, ten isolates were found to share identities with *C. indologenes*. FAME analysis revealed that 13 isolates shared similarities with species of *Chryseobacterium*. Sequencing results of partial 16S rRNA gene or the 16S-23S ISR region revealed that all 13 *Chryseobacterium* isolates shared 100% identities with each other. These results suggest that both API and

FAME could be used to identify bacteria at genus level. However, to have an exact speciation, molecular identification using sequence information might be necessary when API and FAME results are not consistent.

Virulence study by bath immersion revealed that some *C. indologenes* could kill 10–20% yellow perch at the concentration of *c.* 6×10^7 CFU ml^{-1} for 1 h under normal feeding condition. However, some *C. indologenes* isolates failed to kill any fish at the immersion condition we used in the study. Therefore, it is currently unclear whether higher concentrations and/or prolonged exposure might increase the mortality. It was reported that a prolonged exposure (18 h) of Atlantic salmon to both *C. piscicola* and *Flavobacterium psychrophilum* at a low concentration of $2{\cdot}5 \times 10^6$ CFU ml^{-1} killed 100% fish at 28 days post-exposure (Ilardi and Avendaño-Herrera 2008), although neither *C. piscicola* nor *Fl. psychrophilum* itself killed any fish at concentration of 5×10^6 CFU ml^{-1}. In the summer of 2012, a disease outbreak occurred again in that farm. Sequencing results of 16S rRNA revealed that all diseased yellow perch during the summer outbreak had *Fl. columnare* (T. Welch, NCCCWA, USDA-ARS, Leetown, VA, personal communication). Whether a combination of *Fl. columnare* and *C. indologenes* will kill more yellow perch merits further study.

At a high concentration of 6×10^7 CFU ml^{-1} after bath immersion for 1 h, the *C. indologenes* isolates only killed 10–20% yellow perch. It was previously reported that the presence of extracellular products (ECPs) in the old culture media could inhibit the virulence of *Aeromonas hydrophila* to channel catfish (Pridgeon and Klesius 2011). At a concentration of $1{\cdot}65 \times 10^8$ CFU ml^{-1}, a highly virulent *Aer. hydrophila* strain AL09-73 without its ECPs killed 100% of catfish fingerlings within 2 h by bath immersion (Pridgeon and Klesius 2011). However, in this present study, when the old culture media of *C. indologenes* was replaced with fresh media, the virulence of *C. indologenes* was not affected (data not shown). The LD_{50} and LD_{95} values of *C. indologenes* isolate 4 to yellow perch by intraperitoneal injection were $1{\cdot}5 \times 10^8$ and $3{\cdot}2 \times 10^8$ CFU per fish, respectively. We previously (Pridgeon and Klesius 2011) reported that the LD_{50} and LD_{95} values of *Aer. hydrophila* isolate AL09-73 to channel catfish ($4{\cdot}6 \pm 1{\cdot}3$ g) by intraperitoneal injection were 2×10^2 and $9{\cdot}4 \times 10^2$ CFU per fish, respectively. Taken together, these results suggested that *C. indologenes* isolated from the diseased yellow perch was not highly virulent by it alone. A Finland isolate of *C. piscicola* was reported to be only moderately virulent to Atlantic salmon (Ilardi *et al.* 2010). However, species of *Chryseobacterium* are consistently isolated from diseased fish in different countries, including *C. scophthalmum* from diseased turbot in Scotland (Mudarris *et al.* 1994), *C. shigense* from diseased rainbow trout in Spain (Zamora *et al.* 2012) and *C. piscicola* from diseased Atlantic salmon in Chile (Ilardi and Avendaño-Herrera 2008; Ilardi *et al.* 2009), suggesting that *Chryseobacterium* species could become pathogenic to fish under certain conditions.

In summary, 15 Gram-negative bacterial isolates were recovered from the skin lesions of diseased yellow perch. Biochemical API 20NE tests revealed that ten isolates shared homologies with *C. indologenes*. FAME analysis revealed that 13 isolates shared similarities with species of *Chryseobacterium.* Sequencing results revealed that all 13 *Chryseobacterium* isolates identified by FAME shared 99% identities with the 16S rRNA sequence of *C. indologenes* strain B7 (GenBank HQ259684). Based on 16S-23S rRNA ISR sequence, the 13 isolates only shared 88% identities with the 16S-23S ISR sequence of *C. indologenes* strain ATCC 29897 (GenBank EU014570). T-coffee multiple sequence alignment revealed that the partial 16S rRNA or 16S-23S ISR sequence of the 13 isolates shared 100% identities with each other. When yellow perch were exposed to the 15 isolates by bath immersion at concentration of *c.* 6×10^7 CFU ml^{-1} for 1 h, only *C. indologenes* isolates killed 10% to 20% of fish, whereas other isolates were avirulent. When yellow perch was exposed to *C. indologenes* by intraperitoneal injection, mortality was dose dependent, with LD_{50} and LD_{95} values of $1{\cdot}5 \times 10^8$ and $3{\cdot}2 \times 10^8$ CFU per fish, respectively. This is the first report on the isolation of *C. indologenes* from diseased yellow perch. Virulence studies suggest that *C. indologenes* could become pathogenic to yellow perch.

Acknowledgements

We thank Drs Dunhua Zhang (USDA-ARS) and Victor Panangala (USDA collaborator) for critical reviews of the manuscript. We thank Dr Brian Scheffler and Fanny Liu (USDA-ARS-Catfish Genetics Research Unit) for their excellent sequencing work. We thank Beth Peterman (USDA-ARS) for her excellent technical support. This study was supported by the USDA/ARS CRIS project no. 6420-32000-024-00D. The use of trade, firm or corporate names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

References

de Beer, H., Hugo, C.J., Jooste, P.J., Willems, A., Vancanneyt, M., Coenye, T. and Vandamme, P.A. (2005)

Chryseobacterium vrystaatense sp. nov., isolated from raw chicken in a chicken-processing plant. *Int J Syst Evol Microbiol* **55**, 2149–2153.

de Beer, H., Hugo, C.J., Jooste, P.J., Vancanneyt, M., Coenye, T. and Vandamme, P. (2006) *Chryseobacterium piscium* sp. nov., isolated from fish of the South Atlantic Ocean off South Africa. *Int J Syst Evol Microbiol* **56**, 1317–1322.

Benmalek, Y., Cayol, J.L., Bouanane, N.A., Hacene, H., Fauque, G. and Fardeau, M.L. (2010) *Chryseobacterium solincola* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **60**, 1876–1880.

Bhuyar, G., Jain, S., Shah, H. and Mehta, V.K. (2012) Urinary tract infection by *Chryseobacterium indologenes*. *Indian J Med Microbiol* **30**, 370–372.

Cho, S., Lee, K.S., Shin, D., Han, J., Park, K.S., Lee, C.H., Park, K.H. and Kim, S.B. (2010) Four new species *of Chryseobacterium* from the rhizosphere of coastal sand dune plants, *Chryseobacterium elymi* sp. nov., *Chryseobacterium hagamense* sp. nov., *Chryseobacterium lathyri* sp. nov. and *Chryseobacterium rhizosphaerae* sp. nov. *Syst Appl Microbiol* **33**, 122–127.

Decostere, A., Haesebrouck, F. and Devriese, L.A. (1997) Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish. *J Clin Microbiol* **35**, 322–324.

Di Tommaso, P., Moretti, S., Xenarios, I., Orobitg, M., Montanyola, A., Chang, J.M., Taly, J.F. and Notredame, C. (2011) T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. *Nucleic Acids Res* **39**, 13–17.

Hantsis-Zacharov, E., Senderovich, Y. and Halpern, M. (2008) *Chryseobacterium bovis* sp. nov., isolated from raw cow's milk. *Int J Syst Evol Microbiol* **58**, 1024–1028.

Hsueh, P.R., Teng, L.J., Yang, P.C., Ho, S.W., Hsieh, W.C. and Luh, K.T. (1997) Increasing incidence of nosocomial *Chryseobacterium indologenes* infections in Taiwan. *Eur J Clin Microbiol Infect Dis* **16**, 568–574.

Ilardi, P. and Avendaño-Herrera, R. (2008) Isolation of *Flavobacterium*-like bacteria from diseased salmonids cultured in Chile. *Bull Eur Assoc Fish Pathol* **28**, 176–185.

Ilardi, P., Fernandez, J. and Avendano-Herrera, R. (2009) *Chryseobacterium piscicola* sp. nov., isolated from diseased salmonid fish. *Int J Syst Evol Microbiol* **59**, 3001–3005.

Ilardi, P., Abad, J., Rintamäki, P., Bernardet, J.F. and Avendaño-Herrera, R. (2010) Phenotypic, serological and molecular evidence of *Chryseobacterium piscicola* in farmed Atlantic salmon, *Salmo salar* L., in Finland. *J Fish Dis* **33**, 179–181.

Kim, K., Lee, K., Oh, H. and Lee, J. (2008) *Chryseobacterium aquaticum* sp. nov., isolated from a water reservoir. *Int J Syst Evol Microbiol* **58**, 533–537.

Mudarris, M., Austin, B., Segers, P., Vancanneyt, M., Hoste, B. and Bernardet, J.F. (1994) *Flavobacterium scophthalmum* sp. nov., a pathogen of turbot (*Scophthalmus maximus* L.). *Int J Syst Bacteriol* **44**, 447–453.

Notredame, C., Higgins, D.G. and Heringa, J. (2000) T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J Mol Biol* **302**, 205–217.

Park, S., Kim, M., Baik, K., Kim, E., Rhee, M. and Seong, C. (2008) *Chryseobacterium aquifrigidense* sp. nov., isolated from a water-cooling system. *Int J Syst Evol Microbiol* **58**, 607–611.

Pridgeon, J.W. and Klesius, P.H. (2011) Virulence of *Aeromonas hydrophila* to channel catfish *Ictaluras punctatus* fingerlings in the presence and absence of bacterial extracellular products. *Dis Aquat Organ* **95**, 209–215.

Vandamme, P., Bernardet, J., Segers, P., Kersters, K. and Holmes, B. (1994) New perspectives in the classification of the *Flavobacteria*: description of *Chryseobacterium* gen. nov., *Bergeyella* gen. nov., and *Empedobacter* nom. rev. *Int J Syst Bacteriol* **44**, 827–831.

Yoon, J., Kang, S. and Oh, T. (2007) *Chryseobacterium daeguense* sp. nov., isolated from wastewater of a textile dye works. *Int J Syst Evol Microbiol* **57**, 1355–1359.

Zamora, L., Vela, A.I., Palacio, M.A., Domínguez, L. and Fernández-Garayzábal, J.F. (2012) First isolation and characterization of *Chryseobacterium shigense* from rainbow trout. *BMC Vet Res* **8**, 77–82.